Importance of oligo-(*R*)-3-hydroxybutyrates to *S. lividans* KcsA channel structure and function

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In the polyphosphate model of the *Streptomyces lividans* potassium channel KcsA, four polypeptides, each covalently modified by oligo-(*R*)-3-hydroxybutyrates (cOHB), surround a core molecule of inorganic polyphosphate (polyP). PolyP attracts, binds, and conducts K^+ in response to an electrochemical stimulus whilst the polypeptides govern access to polyP and regulate its selectivity. However, the role of cOHB has remained uncertain. Here we identify cOHB-conjugated residues in the ion pathway, S102 and S129, and mutate them to determine the influence of cOHB on channel properties. We find that the mutations have no discernible effect on tetramer formation or tetramer stability; however, cOHB influences polyP incorporation and/or retention, *i.e.* single mutants S102G and S129G contain $\sim$1/3 and double mutant S102G:S129G $\approx$ 1/2 as much polyP as wild-type. Moreover, planar lipid bilayer studies of wild-type and mutant proteins indicate that cOHB has a critical effect on channel function: at positive potentials, only $\sim$5% of S102G and S129G currents and $<$1% of S102G:S129G currents consist of well-structured channels; at negative potentials, S102G and S129G display only irregular conductance and S102G:S129G exhibits no conductance whatsoever. The results indicate that cOHB facilitates the incorporation and/or retention of polyP and plays a critical role in maintaining the flexible polyP molecule in an optimal transbilayer orientation for efficient K^+ transport.

Introduction

Streptomyces lividans, KcsA, is an important model for potassium channel structure and function. This 160 amino acid polypeptide oligomerizes in a tetramer to form a highly selective potassium channel. In a landmark paper by Schrempf *et al.*,[1] the *kcsA* gene was cloned, the KcsA protein was purified and reconstituted, and its electrophysiological behavior was described in giant liposome–protoplast vesicles with a K^+ gradient at pH 7.2. However, successive investigators found that KcsA functions in planar lipid bilayers with a strong pH gradient, pH <5 at the intracellular side.[2,3] These latter studies provided the basis for the prevailing view of KcsA as a pH-gated channel.[4] Important experimental differences among the above studies are the presence of a K^+ gradient and Mg^{2+} in the observations made at neutral pH and their absence in observations made at acidic pH. Subsequent studies confirmed the importance of both a K^+ gradient $>2:1$ and Mg^{2+} for KcsA channel activity at neutral pH.[5]

Analyses of KcsA by Reusch[6] indicated that the polypeptides are covalently modified by oligo-(*R*)-3-hydroxybutyrates (OHB) and the tetramer contains inorganic polyphosphate (polyP). It was proposed that KcsA is a supramolecular complex in which four polypeptides, each covalently modified by OHB (cOHB), encircle a guest molecule of polyP. This view is supported by energy-filtered electron microscopy (EFTEM) studies which show that polyP strands accumulate at the inner face of the cytoplasmic membranes in wild-type *S. lividans* but not in a *kcsA* mutant strain, indicating coincidence between the presence of the KcsA channel and structured polyP.[7] Since the associations between the host polypeptides and the guest polyP are noncovalent and KpolyP is water-soluble, the retention of polyP in its native conformation within KcsA is difficult but essential if one is to observe the true performance of the channel. It is considered that Mg^{2+}, with its high energy of hydration, helps to retain polyP within KcsA by binding tightly both to the polyP end unit and to water molecules. Loss of polyP during purification regimens may be responsible for the low intracellular pH required for channel activity in artificial systems. Tetramers without polyP would no longer be able to function as K^+ channels at physiological pH but may still be able to conduct protons when a strong proton gradient is applied. In addition, Zakharian and Reusch[8] found that functional reconstitution of recombinant KcsA requires incubation ≥ 26 °C, suggesting that this complex structure must undergo conformational reorganization to attain the native form.

The role of polyP in KcsA has been elucidated. KcsA performance at physiological pH derives from interactions between polyP and the KcsA polypeptides.[9,10] In particular, K^+ selectivity is governed at the cytoplasmic end of the channel by interactions between polyP and the C-terminal arginines of the polypeptides. The role of cOHB in this channel, however, remains unclear. Although complexes of polyP with longer polymers of (*R*)-3 hydroxybutyrate (PHB $\sim$140 units) form ion-selective channels in planar lipid

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bilayers,[11–13] the roles of R-3-HB polymers in these complexes, namely, providing coordination sites for cations and a hydrophilic pathway for polyP through the bilayer, have been largely assumed by the polypeptides in KcsA. Yet the presence of cOHB in KcsA suggests it still plays an significant role. Here we locate binding sites for cOHB in the ion pathway of KcsA, and mutate these sites to determine the effect of cOHB on KcsA structure and function.

Results

Identification of OHB conjugation sites in the ion pathway of KcsA

Western blot and chemical assays have shown that KcsA contains oligo-*R*-3-hydroxybutyrates (OHB).[6] The OHB are presumed to be covalently bound (cOHB) because they are not removed by chloroform extraction. Of particular interest for this study are OHB-conjugated residues in the ion pathway (C-terminal to narrow pore) which may interact with the guest polyP. Hydroxy amino acids are likely covalent binding sites and OHB has previously found to be covalently bound to serine residues in *E. coli* membrane porin OmpA.[14] Consequently, serine residues in the ion pathway, as visualized by the X-ray crystal structure[15,16] and solution NMR,[17] were considered to be potential cOHB binding sites. This segment includes two serines: S102 in the vestibule and S129 in the C-terminal strands (Fig. 1).

The single mutants S102G and S129G and the double mutant S102G:S129G were prepared by site-directed mutagenesis (see Experimental). His-tagged wild-type, S102G, S129F and S102G:S129F were each overexpressed in *E. coli* BL21 cells and purified by Ni agarose chromatography. Cyanogen bromide digestion of KcsA produces two large polypeptides—residues 5–96 (9.8 kDa) and residues 97–154 (6.7 kDa) (Fig. 2A). The 6.7 kDa fragment contains most of the ion pathway including the two serines considered to be putative OHB binding sites. Accordingly, wild-type and mutant KcsAs were each digested with CNBr. Western blot assay of the digests using anti-OHB IgG indicates that both 9.8 kDa and 6.7 kDa fragments from wild-type KcsA and single mutants S102G and S129G are modified by cOHB (Fig. 2B, lanes 1, 2, and 3). However, the 6.7 kDa fragment of the double mutant S102G:S129G displays no reaction (Fig. 2B, lane 4). This result indicates that both S102 and S129 are modified by cOHB.

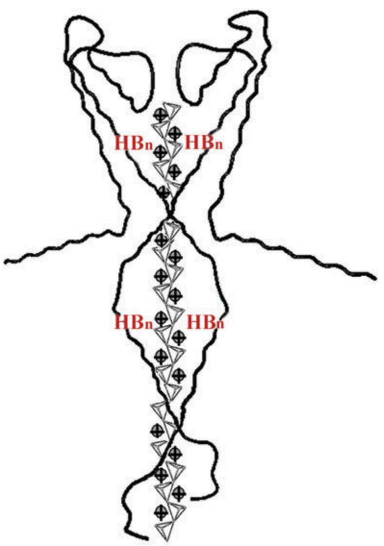

Fig. 1 Putative position of cOHBs within KcsA polypeptides. Model shows two of the four polypeptides of KcsA from RCSB Protein Data Bank (1F6G). The positions of serines S102 and S129, and arginines R159 and R160 are as determined by Cortes *et al.*[17] The serines are modified by cOHB (HBn).

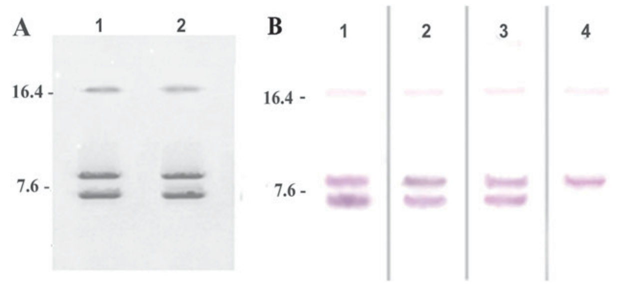

Fig. 2 (A) SDS-PAGE (16.5%) gels of CNBr digests of KcsA (see Experimental). Lane 1, wild-type; lane 2, S102G:S129G. (B) Western blots of KcsA digests probed with anti-OHB_8-IgG. Lane 1, wild-type; lane 2-S102G; lane 3, S129G; lane 4, S102G:S129G.

Influence of cOHB on tetramer stability

KcsA tetramers are very stable. The tetramers are not dissociated into monomers by 2% SDS at room temperature; dissociation occurs only when the protein is heated in 2% SDS. It was considered that cOHB molecules in the conduction pathway may influence tetramer formation and/or tetramer stability. The ratio of tetramers to monomers in preparations of wild-type and mutant KcsAs were compared by observing their mobility on SDS-PAGE gels when they were loaded unheated in 2% SDS and again after heating in 2% SDS in a boiling water-bath for 3 minutes. Wild-type and mutants all migrated as tetramers of $\sim$65 kDa when unheated before loading and as monomers of $\sim$19 kDa after heating (Fig. 3). This result indicates that cOHB in the ion pathway has no significant influence on tetramer formation or stability.

Influence of cOHB on incorporation and/or retention of polyP

PolyP was first detected in wild-type KcsA by its metachromatic reaction to *o*-toluidine stain.[6] Proteins are stained blue but polyPs of >5 units exhibit metachromasy, *i.e.* they shift the absorption maximum of the *o*-toluidine dye from 507 nm (blue) to 530 nm (violet).[17] The identity of polyP was confirmed by chemical assay and by an enzymatic assay in which polyphosphate kinase is used to transfer terminal phosphates from polyP to [^{14}C]ADP yielding [^{14}C]ATP.[6,18]

The effect of cOHB on the ability of KcsA tetramers to incorporate and/or retain polyP was now examined. PolyP was extracted from wild-type and mutant KcsAs (see Experimental) and the polyP content was compared using a semi-quantitative chemical assay in which polyP is hydrolyzed to phosphate and phosphate is then determined with ammonium molybdate, Malachite green reagent (see Experimental). The results indicated that the wild-type KcsA contained

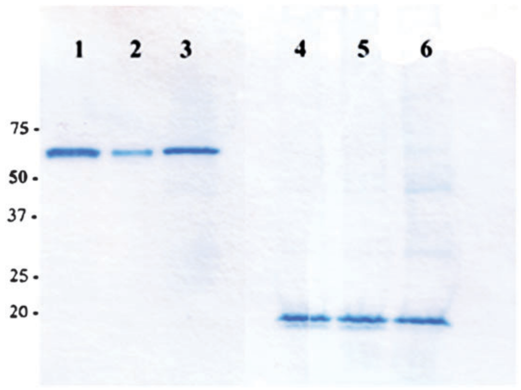

Fig. 3 Effect of cOHB on tetramer formation. SDS-PAGE (12%) of wild-type and mutant KcsA stained with Bio-Safe Coomassie. Loaded without heating: lane 1, S102G; lane 2, S129G; lane 3, S102G:S129G. Loaded after heating in 2% SDS: lane 4, S102G; lane 5, S129G; lane 6, S102G:S129G.

~22 μg polyP/mg KcsA (~25 residues) and that the single-mutants and double-mutant proteins contained ~1/3 and ~1/2 less polyphosphate, respectively, than the wild-type.

A functional indicator of the presence of polyP in KcsA is the isoelectric point (pI) of the tetramers. The theoretical pI (UniProtKB) of KcsA polypeptides is 10.3. Wild-type KcsA monomers exhibit a pI of 10.3; however, wild-type KcsA tetramers display a near neutral pI (6.5–7.5), which is attributable to the presence of the guest polyanion, polyP.[6] Loss of polyP from the tetramer can thus be monitored by observing pI. Accordingly, tetramers of wild-type KcsA and S102G, S129G and S102G:S129G mutants were examined on isoelectric focusing gels (pI 3–10). All displayed a band near neutral pI signifying they contained tetramers with polyP (Fig. 4). However, a fraction of the single-mutant and double mutant proteins remained in the pockets, indicating a pI > 10.0. SDS-PAGE of the same samples ruled out the presence of monomers. Analytical isoelectric focusing on pI 3–10 gels showed similar results except that in this system the high pI fraction moves off the gel and is not visible when focusing is completed.[6] These studies confirm that wild-type tetramers are more likely to contain polyP than mutant tetramers and indicate that cOHB assists in the incorporation and/or retention of polyP within the KcsA tetramer.

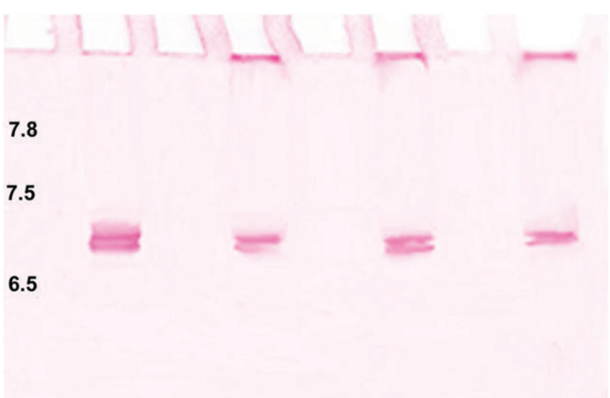

Fig. 4 Isoelectric focusing of wild-type and mutant KcsAs. Proteins were separated on a precast polyacrylamide gel (3–10) (Bio-Rad) and stained with Bio-Rad IEF stain (see Experimental). Lane 1, wild-type; lane 2, S102G; lane 3, S129G; lane 4, S102G:S129G.

Planar lipid bilayer observations of cOHB-mutant KcsAs

The effect of cOHB on channel properties of KcsA was examined *via* single-channel observations in planar lipid bilayers. This method allows one to directly observe channel structure and channel conductance. The purified wild-type and mutant proteins were each reconstituted into liposomes of synthetic POPC : POPE : POPG (3 : 3 : 1) and incorporated into planar bilayers of the same lipids between 10 : 1 gradient solutions of KCl in 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$. Recordings (2 to 10 minutes each) were made at positive and at negative potentials for at least two separate preparations of each protein.

Under the above conditions, wild-type KcsA, as in previous observations,[9,10] displayed continuous well-structured channels of ~150 pS at positive potentials and ~12 pS at negative potentials (Fig. 5). However, the S102G and S129G mutants each displayed well-structured channels in brief periods during only ~5% of observations made at positive potentials (a representative observation of a rare well-structured channel is shown for each in Fig. 5); the remaining ~95% of observations consisted only of highly irregular conductance (not shown). The conductance of these channels varied but in all cases was lower than wild-type. At negative potentials, only irregular conductance was observed (Fig. 5). The S102G:S129G mutant displayed well-structured channels of low conductance in <1% of observations (a representative current record of this very rare event is shown in Fig. 5), and highly irregular conductance in >99% of observations made at positive potentials (not shown). No conductance of any kind was ever observed for the double mutant at negative potentials. These results suggest that the absence of cOHB at one or both serines negatively affects the alignment of polyP within KcsA. Although a fraction of the mutant channels do not contain polyP (Fig. 4), the data indicate that even when polyP is present, it is rarely in a functional conformation within the mutant polypeptides.

The low probability of forming well-structured channels made it impossible to obtain $1/V$ curves for the mutant proteins. However, at +75 mV, the S102G mutant channels had ~10% of the current amplitude of the wild-type with trial openings to ~85% of the current amplitude of the wild-type, the S129G mutant channels had ~50% of the current amplitude of the wild-type, and the S102G:S129G mutant channels had ~30% of the current amplitude of the wild-type.

Discussion

Here we find that serines in the ion pathway of KcsA, S102 and S129, are modified by cOHB and that this modification facilitates the incorporation and/or retention of polyP within the tetramers and is critical to the functional transbilayer alignment of polyP.

cOHB and polyP have not been observed in numerous studies of the structure of the membrane portion (residues 23–119), cytoplasmic portion (residues 129–158) and full-length

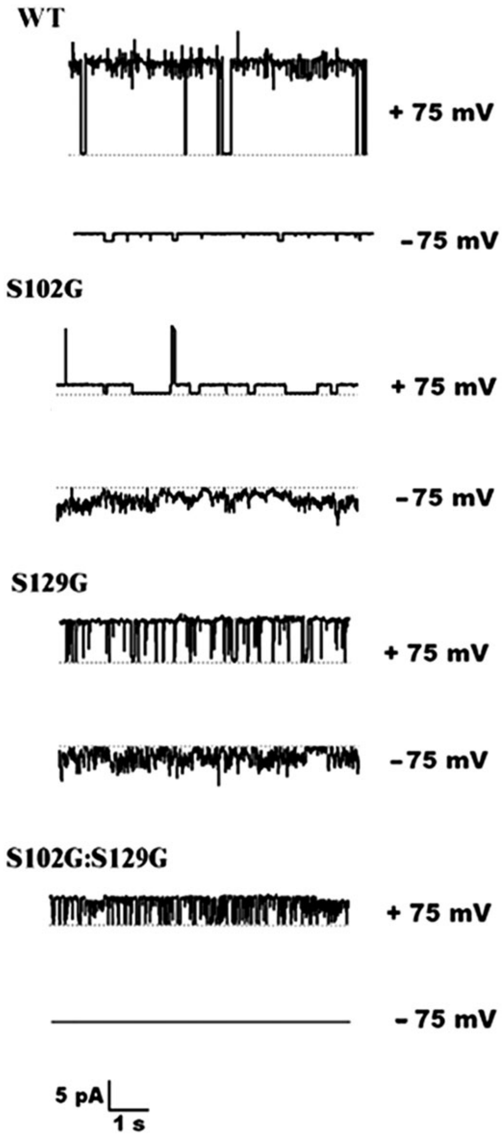

Fig. 5 Representative single-channel currents for wild-type and mutant KcsAs. Wild-type KcsA and mutants S102G, S129G and S102G:S129G were reconstituted into liposomes of POPC : POPE : POPG (3 : 3 : 1), incubated at 42 °C for 1 h, and incorporated into planar lipid bilayers of the same lipids between aqueous solutions of 200 mM KCl, 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$ *cis* and 20 mM KCl, 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$ *trans* at 22 °C. Dotted line indicates the closed state.

polypeptides of KcsA obtained by many physical methods.[15–17,21–24] Those analytical studies have provided a clear view of the arrangements of the amino acid residues in the KcsA channel. However, the methods used to purify KcsA for those studies do not preserve or detect the supramolecular structure of the native ion channel.[6]

A supramolecular complex is by definition held together by weak forces. PolyP does not form covalent bonds, thus it is held within the vestibule and C-terminal bundles (Fig. 1) by noncovalent interactions in which polyP forms ionic bonds to K^+ which in turn form coordinate bonds to amide oxygens of the polypeptides or ester oxygens of cOHB. These non-covalent interactions are relatively weak and KpolyP is water-soluble,[25] hence it may be lost during protein purification, in the bilayer system (channel rundown?), when segments of the polypeptides are cut off, or when antibody fragments are attached to them as *e.g.* to facilitate crystallization. Although cOHB is covalently attached to serine residues, it has no conspicuous atoms or residues. In addition, it has a highly flexible backbone[26–29] and the ester bonds are subject to hydrolysis; thus, while at least some cOHB will likely remain after purification, it would be an unstructured oligomer of variable length, resembling solvent molecules, lipids, detergents or crystallization aids such as polyethyleneglycol. There is sound evidence for the presence of these homopolymers within KcsA. The existence of a polyanion within the tetramer is signaled by the sharp difference in the theoretical isoelectric point of the monomer and the experimental isoelectric point of the tetramers (Fig. 4).[6] Chemical and enzymatic analyses unambiguously identify the polyanion as polyP.[5–8] Western blot, chemical and enzymatic assays clearly establish the presence of cOHB in KcsA polypeptides (Fig. 2).[5,6,11,12]

As a flexible 'wire' of negatively-charged phosphate units,[25] polyP is uniquely qualified to attract, bind, dehydrate and transport cations. PolyP was first discovered to be a component of ion channels in bacterial membranes where it was found complexed to ~140 unit polymer of R-3-HB.[30,31] In this complex, the interactions between the two homopolymers and cations would be fairly uniform. However, the structures of polypeptides are much more complex and this makes their interactions with KpolyP less consistent, leaving gaps in some regions which allow the flexible polyP molecule to bend and twist, particularly with the application of a voltage pulse. Such activity may explain the irregular conductance or lack of conductance observed with KcsA mutants (Fig. 5). Even when allowing for the absence of polyP in up to half of the mutant tetramers, the extremely low probability of observing well-formed channels by the mutant proteins indicates that polyP alignment is seriously affected by the absence of cOHB. In this regard, the serine residues modified by cOHB are situated at positions in which the polypeptides are distant from the central core of the tetramer and to polyP (Fig. 1). cOHB provides additional coordination sites for K^+ and acts as a gasket in maintaining a functional transbilayer orientation of polyP within the tetramer.

The important contributions of the polypeptides to KcsA function should not be overlooked. In addition to forming a pathway for polyP across the bilayer, the polypeptides play a critical role in the selection of K^+. At the external end, they create a narrow pore that is well-situated to prevent extracellular cations with inward gradients, namely Ca^{2+} and Na^+, from gaining access to the polyP conductor and flowing into the cytoplasm. This narrow opening, which is known as the 'selectivity pore', may more correctly be called the 'barrier pore'. At the channel entry, the terminal arginine residues form a collar of eight positive charges around the terminal phosphate residue, which has an effect similar to lowering pH in mitigating its divalent negative charge. As a result, polyP loses its preference for divalent cations, and its selectivity for K^+ over Mg^{2+}, the major intracellular competitor,[10,11] is increased. By their structure and composition, the KcsA polypeptides fashion polyP into a K^+ conducting instrument at physiological pH.

Our studies suggest a new paradigm for channel function. They indicate that the KcsA channel is regulated by electrostatic rather than by mechanical forces. Molecular motions certainly occur but mechanical movements of helices, induced by sharp fluctuations in cytoplasmic pH, are not required for opening and closing physical 'gates'. The ionic bonds between polyP and K^+ introduce a new dynamic in which electrostatic forces control the outward flow of K^+. In this view, the ionic bonds between K^+ and the polyP polyanion create the 'gate'. When the components of the electrochemical potential, either individually or in concert, overcome the strength of the ionic bonds, the channel is 'open', *i.e.* K^+ vents through the narrow pore at the external end. When equilibrium is reestablished, the channel is then 'closed', *i.e.* K^+ ceases to flow. Accordingly, the channel functions simply as a K^+ safety valve.

The physical properties of cOHB and polyP, their universal presence in biological cells,[32,33] and their widespread occurrence in ion transporters suggest that they play important roles in transmembrane movement of ions in both prokaryotes and eukaryotes. cOHB and polyP have been found in eukaryotic plasma membranes, mitochondria and microsomes,[34] in the CaATPase pump of human erythrocytes,[35] in the voltage-dependent channel of mitochondria,[36] in porin Omp P5 of *Haemophilus influenzae*,[37] and most recently in the TRPM8 channel of sensory neurons.[38] These studies suggest that cOHB and polyP may be regarded as fundamental constituents of ion transporters.

Experimental

Preparation of KcsA mutants

All mutations were made using the Quik-change™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Wild-type KcsA DNA was used as the template for mutants S102G and S129G, and S129G DNA was used as the template for double mutant S102G:S129G. All mutations were confirmed by DNA sequencing at the MSU Genomics Center.

Purification of *S. lividans* KcsA wild-type and mutants

Wild type KcsA, His-tagged at the C-terminal was cloned into pQE60 (courtesy of C. Miller), transformed into *E. coli* BL21 cells (Novagen), cultured in Terrific Broth (12 g tryptone, 24 g yeast extract, 4 ml glycerol per litre dH_2O) and overexpressed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Calbiochem) to a final concentration of 1 mM. The cells were harvested by low-speed centrifugation, resuspended in 1/50 volume of All Purpose Buffer (APB) (50 mM Hepes, 1 M KCl, 10 mM $MgCl_2$, 250 mM sucrose). Lysozyme (20 μg ml^{-1}), DNase (20 μg ml^{-1}), and protease inhibitors leupeptin (9.5 μg ml^{-1}), pepstatin (14 μg ml^{-1}) and PMSF (0.17 mg ml^{-1}) (Sigma) were added and the mixture was incubated for 30 min at 4 °C. Following ultrasonication (2 × 1 min), the suspension was centrifuged in a Beckmann type 50.2 rotor at 40 000 rpm for 50 min. The pellet was resuspended in 10 ml of APB buffer and 50 μl Triton X-100 and 75 mg *n*-dodecyl-β-maltoside (DDM) (Calbiochem) were added. The suspension was again centrifuged at 35 000 rpm for 45 min. The supernatant was purified by Ni-affinity chromatography as described by the manufacturer (Qiagen). The eluted proteins formed single bands at MW ≈ 65 kDa on SDS-PAGE gels when unheated, corresponding to the tetrameric form, and they were converted to the monomeric form, ~19 kDa, when heated in 2% SDS. Protein concentrations were determined using the detergent-compatible (DC) assay (Bio-Rad).

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Digestion with cyanogen bromide

Purified KcsA wild-type protein was dissolved in 70% formic acid and digested o/n with cyanogen bromide (CNBr). After removal of excess CNBr and solvent and desalting, the protein fragments were dissolved in 2% SDS, separated on 16.5% SDS-PAGE gels, and transferred to supported nitrocellulose membranes (Bio-Rad) for Western blot analysis or to sequencing grade PVDF membrane for N-terminal Edman sequencing.

Determination of cOHB

Peptides 97–154 from CNBr digestion of wild-type mutant KcsA were tested for the presence of cOHB by Western blot immunoassay using anti-OHB_8-IgG.[6] Second antibody was goat anti-rabbit alkaline phosphatase conjugate in the same buffer. Color development was performed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) (Bio-Rad). cOHB was also determined by a chemical assay as previously described.[40] Briefly, KcsA (~200 μg) was precipitated with ice-cold 95% ethanol and dried by lyophilization. Concentrated sulfuric acid was added and the sample was heated at 120 °C for 40 min. The resulting crotonic acid was extracted and chromatographed on an Aminex-HPX-87H ion exchange organic acid analysis column (Bio-Rad; 4.1 × 250 mm) using 0.014 N H_2SO_4 as eluent. Standards were R-3-HB (Sigma) and poly-(*R*)-3-hydroxybutyrate (PHB, Sigma). The crotonic acid peak was identified by its elution time, UV absorption curve, and mass spectrum, and concentrations were estimated by comparison of the peak area with those of crotonic acid standards.

Determination of polyP

KcsA was precipitated with ice-cold acetone and incubated at −20 °C o/n. The precipitate was washed with methanol : acetone (2 : 1) (3×) and acetone (2×) to remove phospholipids and then dried. Phosphate was determined with ammonium molybdate, Malachite green reagent before and after hydrolysis in 1 N HCl for 15 min in a boiling water bath[39] using $polyP_{35}$ (Sigma) as standard.

To estimate polyP size, polyP was extracted from KcsA wild-type and mutants using an adaptation of the method of Crooke *et al.*[19] Briefly, KcsA (500 μg, 1 ml) was incubated with proteinase K (50 μg) at room temperature for 45 min. PolyP5 (10 μg; Sigma) was added as a carrier and the mixture was extracted with $\frac{1}{2}$ volume of phenol : chloroform (1 : 1) equilibrated with 0.1 M ammonium acetate. The sample was thoroughly mixed and the phases were separated by centrifugation at low speed. The aqueous layer was retained and the organic phase was extracted 3× with 10 mM EDTA, pH 7.2. The pooled aqueous layers were back extracted 1× with $\frac{1}{2}$ vol. 1 : 1 phenol : chloroform and then 3× with chloroform. Traces of chloroform were removed with a stream of N_2 gas. The concentrated samples were examined on 15% acrylamide gels (19 : 1 acrylamide : bisacrylamide) (Bio-Rad) using Tris/borate/EDTA (90/90/2.7 mM, pH 8.3) as buffer and brom phenol blue (BPB) and xylene cyanol (XC) as standards.[20] The gel was stained with 0.05% *o*-toluidine blue, 25% methanol, 1% glycerol and destained with 25% methanol, 5% glycerol. BPB and XC migrate to positions corresponding to ~35 and ~72 polyP residues, respectively.

Determination of isoelectric point (pI)

Isoelectric point was determined using 3–10 IEF Ready gels (Bio-Rad). Sample buffer was 50% glycerol; cathode buffer was 20 mM lysine, 20 mM arginine (Bio-Rad); anode buffer was 7 mM phosphoric acid (Bio-Rad). The gel was stained with Coomassie Brilliant Blue R250, crocein scarlet (Bio-Rad IEF stain) and destained with 40% methanol, 10% acetic acid. IEF standards were from Bio-Rad.

Isoelectric points were also determined by analytical focusing on 0.4 mm gels of 5% acrylamide/bisacrylamide (33.7 : 1), 10 mM *n*-dodecyl-β-D-maltoside, 5% 3–10 ampholytes on gel support film using a Bio-Rad model 111 Mini IEF cell.[6] Voltages were stepped from 100 to 450 V for a total of 90 min. Stain and destain were as above.

Planar lipid bilayer measurements

Purified KcsA tetramer was reconstituted into liposomes of synthetic 1-palmitoyl, 2-oleoyl phosphatidylcholine (POPC), synthetic 1-palmitoyl, 2-oleoyl phosphatidylethanolamine (POPE), and synthetic 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) (3 : 3 : 1) (Avanti Polar Lipids) and incubated for >1 h at 42 °C. Planar lipid bilayers were formed from a solution of POPC : POPE : POPG (3 : 3 : 1) in *n*-decane (Aldrich). The lipid solution was used to paint a bilayer on an aperture of ~150 μm diameter in a Delrin cup (Warner Instruments, Hamden, CT) between aqueous bathing solutions of 200 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.4 (*cis*)/20 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.4 (*trans*). All salts were ultrapure (>99%) (Aldrich). Bilayer capacitances were in the range of 25–50 pF.

Recording and data analysis

Unitary currents were recorded with an integrating patch-clamp amplifier (Axopatch 200A, Axon Instr.). The *cis* compartment (voltage command side) was connected to the CV 201A head stage input, and *trans* compartment was held at virtual ground *via* a pair of matched Ag–AgCl electrodes connected to the solutions by an agar bridge containing 3 M KCl. Currents through the voltage-clamped bilayers (background conductance < 1–2 pS) were low-pass-filtered at 10 kHz (−3 dB cutoff, Bessel type response) and recorded after digitization through an analog-to-digital converter (Digidata 1322A, Axon Instr.). Data were filtered through an eight-pole Bessel filter (902LPF, frequency devices) and digitized at 1 kHz using pClamp9 software (Axon Instruments). The data were averaged from recordings of at least two separate preparations of each protein. Each recording was 2 to 10 min long. The concentration gradient was created and the junction potential offset was compensated before membrane painting. Nernst potentials were calculated using ion activities.[41]

Conclusion

cOHB in the ion pathway of the KcsA channel stabilizes the orientation of the flexible guest polyP molecule within the tetramer. cOHB molecules occupy spaces between the polypeptides and polyP and form coordinate bonds to K^+ ions bound to polyP, thereby facilitating the incorporation and/or retention of polyP and maintaining a favorable transbilayer orientation of polyP for efficient K^+ conductance.

Acknowledgements

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